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We take this opportunity to express our gratitude to the Maharaja Sayajirao University of Baroda for a very generous donation given towards the cost of the printing and publication of this journal for the year 1956-'57.

CONTENTS

ON SOME HISTOLOGICAL FEATURES OF THE TEGUMENTAL GLANDS IN *PENAEUS INDICUS, M.Ed.**

A. KRISHNAKUMARAN †

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SEVERAL types of tegumental glands have been described in decapod crustaceans (Farkas, 1927). To these glands have been attributed conspicuously divergent functions. Yonge (1932) contended that the tegumental glands in *Homarus*, secrete a substance which spreads itself over the surface of the endocuticle (which he termed "Chitin") forming a uniform, homogeneous layer, the epicuticle ("Cuticle" according to his terminology). Drach (1939), however, showed that the epicuticle in *Maia squinado* is formed earlier than the endocuticle and is laid down by the general epithelium. Again, the tegumental glands in *Carcinus maenas* are responsible for the secretion of an oxidase (phenolase) which is transported to the cuticle (Krishnan, 1951). Several other functions such as the secretion of salivary juices (Vitzou, 1881), of a fluid which assists in moulting (Farkas, 1927), of egg membranes and statocysts (Yonge, 1935; Lang and Yonge, 1938) have been attributed to these glands. Dennell (1947) concluded that, in view of their close association, with the cuticle and the periodicity they exhibit with the moult cycle, the tegumental glands must be connected with the formation or the moulting of the cuticle. It may be mentioned that in certain insects such as *Rhodnius and Tenebrio*, the dermal glands (the homologues of the crustacean tegumental glands in insects) secrete a constituent layer of the epicuticle, namely, the cement layer (Wigglesworth, 1947 and '48).

An attempt has been made in this study to describe the structure of the tegumental glands and note their appearance in the moult cycle of *Penaeus indicus*.

Material and Methods

In the present study, *Penaeus indicus* collected from the Cooum river at Madras was used. The Material was fixed in 5% formaldehyde

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* Forms part of a thesis submitted to the University of Madras for the degree of Master of Science.

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in 0.9% saline. Dioxane was used for purposes of dehydration so as to avoid hardening. The material was embedded in celloidin according to Peterfi's double embedding method. Fresh frozen sections were taken when it was necessary to determine the chemical nature of the constituents of the glands and gland ducts. Mallory's triple and haematoxylin and eosin were used for staining.

Structure of the Glands

Although every cell underlying the cuticle is glandular in the sense that each of them elaborates and secretes some constituent of the cuticular material (Richards, 1951), there are some specialised glands interspersed with the cells of the chitogenous epithelium, called the tegumental glands. These glands in *Penaeus indicus* are multicellular structures the ducts of which pass through the cuticle and open on the outside. These glands occur either in the chitogenous epithelium or lie below it in the body cavity. The glands when located in the body cavity are surrounded by a thin membranous sheath probably derived from the basement membrane as in *Astacus* (Farkas, 1927) and *Homarus* [Yonge, 1932]. Apart from the difference in the location of the glands they differ in structural features as well. Based on the structural characters three distinct types of tegumental glands are distinguishable in *Penaeus indicus*, namely (1) the spherical, (2) the flask-shaped and (3) the club-shaped glands.

The first variety occurring under the chitogenous epithelium of the tergites only in the middle intermoult stages, is spherical in shape and multicellular in constitution being composed of about ten compactly arranged glandular cells (Fig. 1). The entire structure measures about 50μ in diameter, but smaller ones are also noticed. These structures are bounded by a sheath of connective tissue. A large duct formed by the joining of smaller collecting tubules from each of the individual cells of the gland as those described in *Astacus fluviatilis* (Farkas, 1927), starts from the centre of the gland. Each gland cell has a round nucleus located peripherally and the cytoplasm is finely granular and non-vacuolated. The duct, which stains red with Mallory's triple and blue with haematoxylin starts from the gland and runs a straight course passing through the chitogenous epithelium and cuticle to open on the surface of the cuticle. Further these ducts show a positive reaction with Sudan III and Sudan black B. Such glands seem to be present not only under the tergite cuticle but also in the stomodaeal and proctodaeal parts of the gut.

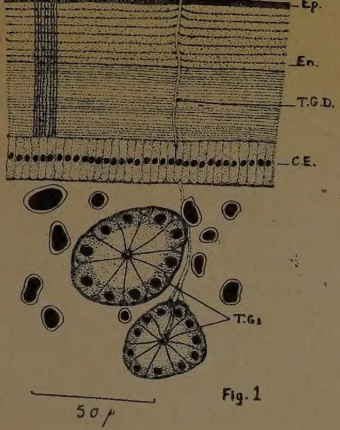


Fig. 1. Section passing through the middle intermoult integument from the tergite showing the spherical glands.

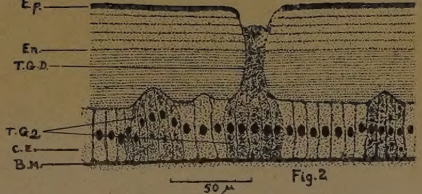


Fig. 2. Section of the premoult integument showing the flask-shaped glands

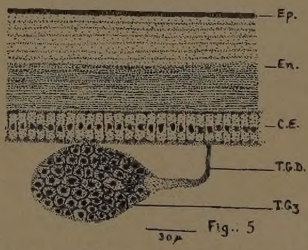
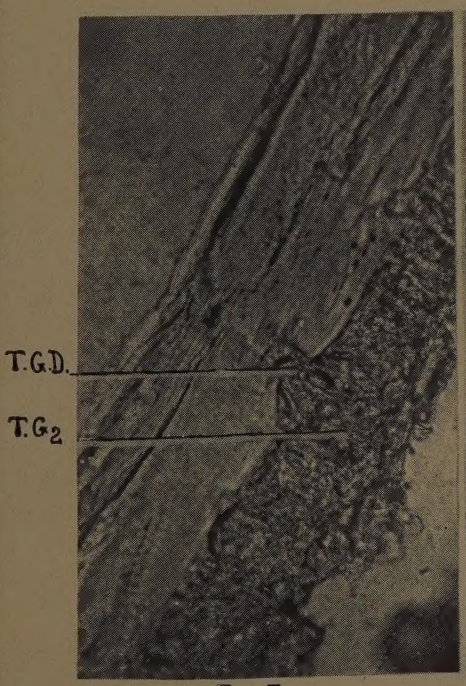


Fig. 5. The same as in Fig. 4 (The duct shown upto the epithelium only.)



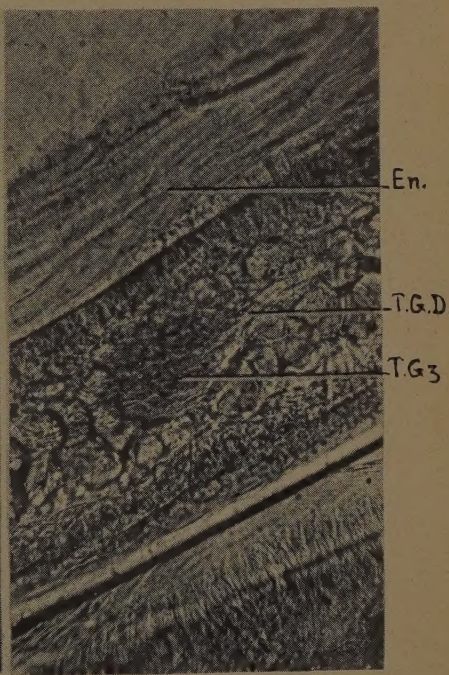


Fig. 3. Photomicrograph of the same as in Fig. 2

Fig. 4. Photomicrograph of the premoult integument to show the club-shaped gland.

B. M. Basement membrane.
C. E. Chitogenous epithelium.
En. Endocuticle.
Ep. Epicuticle.
T. G1. Spherical glands.
T. G2. Flask-shaped glands.
T. G3. Club-shaped glands.
T. G. D. Tegumental gland duct.

The glands described above, present many features which recall those in *Homarus* (Yonge, 1932) such as their location in relation to the cuticle and chitogenous epithelium, their shape, the features of their constituent cells and the staining reaction of the contents of the gland ducts with Mallory's triple and haematoxylin. With Sudan III the ducts show a positive reaction in both the animals. Similar glands have been described in the oesophagus of *Astacus* by Farkas (1927) who suggested that these glands may secrete a slimy substance which aids in the disintegration of the cuticle.

The second type of glands, namely the flask-shaped variety, is located within the chitogenous epithelium and appears to form a part of it. In sections it is seen to be composed of five to six cells forming a flask-shaped cluster (Figs. 2 & 3). The cells that compose these glands are vesicular with their nuclei located towards the proximal end. The surrounding cytoplasm contains scattered globular inclusions which stain with Sudan dyes. The distal ends of these cells lead into a single, straight, wide duct measuring 8μ in width, which passes through the cuticle to open to the outside at a shallow depression on the cuticle.

The third type of gland is club-shaped and occuring below the epithelium in contact with the old cuticle is found in those preparing for moulting (Figs. 4 & 5). The duct of the gland starts in a direction parallel to the chitogenous epithelium. This gland measures about 103μ in length and 42μ in width and is covered by a thin structureless membrane similar to the one noticed in the spherical glands described above. Probably this membrane is derived from the basement membrane. The glands are composed of compactly arranged cubical cells whose walls are distinct. The nucleus located in the centre of each cell stains deep blue with Delafield's haematoxylin. The duct which leaves this gland from its narrower end passes parallel to the epithelium for a short distance and takes a bend at right angles to the latter, towards the old cuticle (Fig. 5).

Discussion

Glands similar to the spherical type have been described by Yonge (1932) in *Homarus*. But he stated that they occur in the premoult stages and, " at no other stage except immediately before the actual moult are degenerating and newly formed glands seen and at no stage are the signs of secretion so widespread ". From the similarity of the staining properties of the epicuticle and the contents of the ducts (For *Penaeus*

Krishnakumaran, 1955) it was contended that the glands are actively concerned in the formation of the new epicuticle. From the observations made in *Penaeus* it seems unlikely that the tegumental glands, which occur in the intermoult stages when the epicuticle is completely formed, have any part to play in the formation of the epicuticle.

The flask-shaped glands described as located in the chitogenous epithelium are unique and no glands similar to these have been so far described in any crustacean, though glands of similar constitution have been described in relation to the cuticle of an insect, the adult male *Tenebrio* (Wigglesworth, 1948). In *Tenebrio* the glands have been noted under the tergites with a newly moulted cuticle. However, wigglesworth (1948) did not attribute any function to these structures. But Blower (1951) who observed in the myriapod, *Schizophyllum*, similar glandular cells located in the epithelium and whose ducts open outside on the surface of the cuticle, suggested that they may secrete lipids, which form a lipidal layer external to the exocuticle. He attributed the positive argentaffin reaction also to be due to lipids, in view of the test not being specific for phenolic substances. Although these glands in *Penaeus* give a positive sudanophilous and argentaffin reaction these glands are noted only in relation with the old cuticle in the premoult stage. Such glands have not been noted in relation to the new cuticle that is being formed at this stage. In view of the above observations it appears unlikely that they contribute to the lipids of the epicuticle. Their function in *Penaeus* is not clear although their appearance at a late intermoult period may suggest a possible role in the dissolution of the cuticle.

The club-shaped glands seen in the premoult stages in relation to the old cuticle bear a close resemblance to those described in the labrum of *Homarus* by Vitzou (1881) in their shape and in being composed of numerous compactly arranged cells. Vitzou (1881) ascribed to such glands a salivary function in view of their abundance in the labrum although he conceded that they may have different functions in the other regions. Their function and the significance of their occurrence in relation to the old cuticle during the premoult stages in *Penaeus* are not clear.

From the insufficient data regarding the chemical nature of the contents of the ducts, it cannot with definiteness be said what their function could be. Nevertheless it is presumable that the three types of gland

have different functions, all or some of which are related to resorption of the various constituents of the cuticle such as the proteins, chitin and lipids.

Summary

From a study of the tegumental glands in *Penaeus indicus* three different types namely the spherical, flask-shaped and club-shaped glands have been described and compared with similar glands known in other arthropods.

Acknowledgement

Grateful thanks are due to Dr. G. Krishnan, Reader in Zoology and Professor C. P. Gnanamuthu, Director, Zoological Research Laboratory, University of Madras, for suggesting the problem and their unfailing guidance during the course of this work. Thanks are also due to Dr. K. P. Rao, Department of Zoology, S. V. University for the help in the preparation of the paper and to the University of Madras for the award of a studentship during the tenure of which this work was carried out.

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MORPHOGENETIC EFFECTS OF ESERINE SULPHATE*

1. The Skeletal Abnormalities

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CHEMICAL teratology has attracted a lot of workers in the past quarter of this century. The interest in it has grown more and more, as, such a study has shown to be of promising value in the study of the normal biochemistry of development. Its far reaching consequence is in pinning down the particular metabolic chain in each case, assuming that an abnormal development results from the deranged metabolism. The accurate assessment of the environmental from the genetic factors involved in the teratogenic processes, has always worried the students of these deranged biochemical processes. To this is added the similarity which these results bear towards the rarely occuring spontaneous malformations. The influence of the teratogenic agents is not merely speculated but seems definite as is observed in the various responses elicited with different groups of experimental animals, and different teratogenic agents.

A very large variety of chemicals (Table 1) have been tried by different investigators in different ways. The work of Ancel (1945) is credited with the use of the largest number of chemicals of diverse composition and pharmacological properties. He tried over ninety different agents out of which he found eserine sulphate and sulphanilamide of definite teratogenic value. In all attempts using chemicals for interference in morphogenesis, differential responses have been elicited in different parts of the body (Landauer, 1954). The results of Ancel and Landauer, confirmed by those of others, show that all these chemicals produce a syndrome of malformations involving the skeleton of the face and the hind limbs, with or without affecting the caudal vertebrae. The effects noted are chiefly skeletal, but in addition a generalised dwarfism of the whole body may result. The parts of the body affected, if at all, depend upon the developmental stage at which the teratogenic agents are used and their dosage. The period of maximum sensitivity to the interference differs with different chemicals.

Below is given a table summarising the results obtained in the more important teratogenic investigations.

* This work was done at the Johns Hopkins school of Medicine, Baltimore U S.A,. under the auspices of the Rockefeller Foundation Fellowship.

TABLE I

Author	Drug	Site of Drug Administration	Experimental Animal	Defects
Stockard, C. R. (1909)	Alcohol	Surrounding Medium	Chick Embryo	Monophthalmia Asymmetrica
Dagg, Charles Patrick and David Karnofsky (1955)	Azaserine	Yolk sac	Chick Embryo	Beak & Limb deformities
J. G. Wilson (1954)	Azodyes	Intraperitoneal	Rat	Ectopia viscerae & Eye defects
Landauer, W. (1952)	Boric Acid	Yolk sac	Chick Embryo	Micromelia, Beak defects & Syndactylysm
Ancel, Paul (1952)	Colchicine	Yolk sac	Chick Embryo	Micromelia and Beak defects
Karnofsky, Ridgway and Patterson (1951)	Cortisone	Yolk sac	Chick Embryo	Growth inhibition
Fraser, F. C. and T. P. Fainstat (1951)	Acetate	Intraperitoneal	Pregnant mice	
Paul Ancel (1945)	Eserine	Yolk sac	Chick Embryo	Micromelia and Parrot beak
Stockard, C. R. (1909)	Ether	Surrounding Medium	Fundulus Heteroclitus	Eye defects
Landauer, W. (1947) Duraiswami, P. K. (1952)	Insulin	Yolk sac	Chick Embryo	Micromelia and beak defects
Sverker Bäckstrom and Tryggve Gustafson (1953)	Lithium	Surrounding Medium	Sea urchin Eggs	Reversed polarity
Stockard, C. R. (1910)	Magnesium	Surrounding Medium	Fundulus Heteroclitus	Cyclopia

TABLE I (Contd.)

Author	Drug	Site of Drug Administration	Experimental Animal	Defects
Dietrich Bodenstein (1947)	Nitrogen-mustard	Surrounding Medium	Amblystoma Embryo	Inhibited Melanophores and Ectoderm
Landauer, W. (1953)	Pilocarpine	Yolk sac	Chick Embryo	Micromelia, Beak defects and Syndactylysm
Franke *et al* (1936) / Landauer (1940)	Selenium	Yolk sac	Chick Embryo	Beak and limb deformities
P. Ancel and Lallemand (1942)	Sulphanilamide	Directly on the Blastoderm	Chick Embryo	Micromelia and beak defects
Zwilling, Edgar and J. T. DeBell (1950)	Sulphanilamide	Yolk sac	Chick Embryo	Micromelia and beak defects
Gray, P. and H. Worthing (1941)	Tetanus Toxin	Yolk sac	Chick Embryo	Brain and cord defects
Karnofsky *et al* (1950)	Thallium	Yolk sac	Chick Embryo	Achondroplasia
Waddington and Carter (1952) / D. L. Gunberg (1954)	Trypan Blue	Intraperitoneal	Rat	Neural tube defects and spina Bifida

On the very reasonable assumption that these chemicals interfere with important metabolic processes, various biochemical studies were undertaken, notably by Zwilling (1951). It was successfully tried (Zwilling, 1949) to reverse the effects of insulin by supplementing Nicotinamide and a-Ketoglutaric acid.

Material and Method

The eggs used for these experiments came from New Hampshire Red and Cornish white flocks. In the experiments by others using sulphanilamide, a solution of it was instilled directly on chick blastoderm in the 48 hours developmental stage. Eserine sulphate was administered into the yolk sac by Ancel and Lallemand. In the present series, the eserine sulphate solution was injected into the air space at the broad end of the egg. The absorption of the solution occurs through the inner shell membrane which is known to be permeable to salt solutions (Salvatori, 1936). The quantity and concentration of the solution found most effective and still leaving the embryos alive up to 10th day, atleast, was 50γ gm. per egg of eserine sulphate in 0.25 ml. of distilled water. Doses of 25γ gm. in the air space and 0.5 mg. in yolk sac were also tried. The former proved less effective and the latter, more lethal. Injections were made from the second to the sixth day of incubation. In each batch of experiment, an equal number of controls were kept after subjecting them to the trauma of drilling a hole at the site of the air space and injecting an equal quantity of distilled water. Because of invariable death after injection if the eggs were left in a vertical position with the blunt end up, it was deemed essential to leave them in a horizontal position for atleast eight hours which was the approximate time taken by 0.25 ml. solution to be completely absorbed. The probable cause of such death could be a blocking of the large respiratory area offered by the air space, since the blunt end of the egg is known to be the richest in number of pores for gaseous exchange (Rizzo, 1899). The usual precautions for temperature and humidity regulation and the mechanical turning of the eggs were observed. In all 300 eggs were used. The results were compiled on the basis of observations on survivors till the 10th day and beyond.

Results

It was found that the period of maximum sensitivity was on the 4th day of incubation. In the present series 16% of the embryos died soon after injection and 24% completely escaped the effect of the drug. The

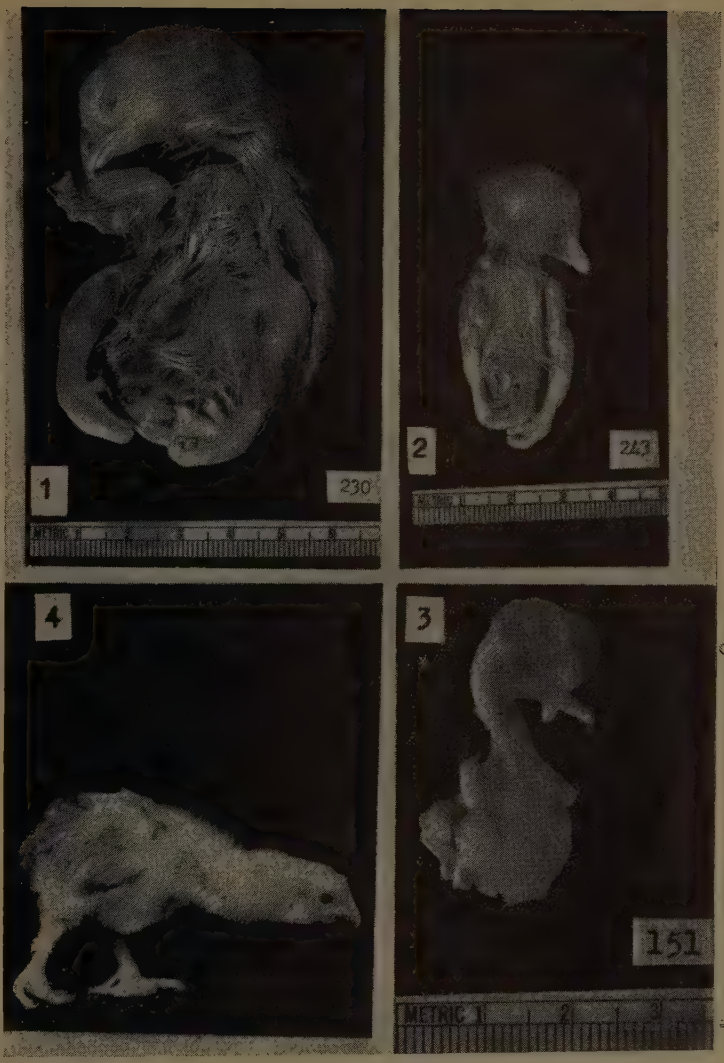

PLATE I

1. Embryo—19 days incubation showing club feet and " parrot beak ".
2. Embryo—15 days incubation showing club feet without any beak deformity.
3. Embryo—10 days incubation showing "cross beaking" with complete suppression of the right eye.
4. Chick —5 months old showing club feet and " parrot beak ".

largest incidence. In all such embryos, the upper beak was directed to the right. This condition sometimes presented itself in association with partial or complete suppression (Pl. II, Fig. 5) of the right eye. The facial skeleton frequently showed " parrot beaking " with shortening of lower jaw and ventral curvature of the upper one. Rarely, the upper jaw showed shortening. " Cross beaking " was never observed in association with the hind limb deformity. In the most affected chicks (Pl. I, Fig. 1) " parrot beaking ", bending of tibio-tarsus and clubbing of the foot were all seen together.

Besides, a generalised dwarfing of the body, an oedematous condition with particular localisation in the lower half of the face was also noticed. Oedema of the lower half of the face was invariably seen to produce curvature of the lower jaw cartilage. This may occur as the only affectation in an embryo or may be in association with other limb abnormalities.

Skiagram of one of the experimental chicks (Pl. II, Fig. 6) showed a complete absence of all appendicular skeleton and in it the axial skeleton was seen only in the anterior half of the body. The vertebral components of the part of the axial skeleton present, could not be demarcated.

Body weight and size was found to be generally below normal in experimental chicks. Quantitative data for these will be included in a further communication on the topic.

Discussion

The route of administration of the drug through the air space of the egg seems to have certain definite advantages over the usual yolk-sac injection. Firstly, it completely avoids any trauma to the embryo and its developing membranes. Such an injury to the embryo may in itself cause malformations, if not death, of the embryo. Secondly, it seems to ensure a more or less complete utilization of the teratogenic agent, simultaneously giving a clue that it affects the developing embryo in a way other than through nutrition, as can be possible in yolk sac injection. Since the living vitelline membrane encourages the entry of salts and impedes their exit (Straub and Hoogerduyn, 1929), the effect of the drug appears to be directly on the blastoderm.

The period of maximum sensitivity, that is, the 4th day of incuba

tion, coincides with the time when the embryo enters into the stage of a rapid growth of its entire body and different organs (Schmalhausen 1926). It is this very critical period in the early development of the chick during which frequent spontaneous deaths occur in embryo due to maladjustments in respiration (Riddle, 1930). The toxic shock of the teratogenic agents administered during the critical period sufficiently accounts for the 16% death rate among embryos just after injection. These embryos were probably in some unknown way, more susceptible than the 60% of embryos showing various abnormalities, and the malformed ones being certainly more sensitive than 24% embryos that resisted and completely escaped the effect.

The affectation of limb bones and facial skeleton points to a disturbance in the growth of cartilaginous primordia of these bones. Due to the briefness of the period during which the disturbance was caused, it cannot be assumed that the effect of the drug was a generalised retardation of growth. On the other hand, the localisation of the effect on the face and hind limbs was probably due to the fact that these were the parts growing at a fast rate during the critical period, and due to their higher metabolic gradient they seem to suffer maximally. The association together of the face *cum* hind limb deformities points to their close metabolic relatedness, atleast during this critical period.

As some embryos escaped the effect even with the maximum tolerated dose administered during the period of maximum sensitivity, it is felt that for an effective response a co-existence of some other single or plural factors is perhaps essential. The geneticists however ascribe it to inherent genetic differences.

Buophthalmos, microphthalmos and anophthalmos, occasionally seen in the experimental embryos, can result from a defective laying down of the cartilaginous orbit. A unilateral orbital defect can reasonably lead to a " cross beak " condition with or without affecting the eye. In this series it was invariably the right eye which showed partial or complete suppression. This unilateral effect could be the result of natural asymmetrical trunk flexure of the embryo bringing the right side of the head to the top and thus exposing it to the effect of the drug more than the left side or that the metabolic gradients of the two eye primordia may be different in this critical period. The " cross beak " condition has never been seen in association with limb deformity.

A generalised oedematous condition of the body has been mentioned by Ancel (1945) but its localisation to the lower part of the face has been observed frequently in these experiments. The oedema is invariably associated with an abnormal bending of the lower jaw cartilage. The oedema may be considered as a part of the generalised syndrome, but the distortion in the lower jaw cartilage can definitely be reckoned as an intermediate stage in the causation of beak deformities.

As mentioned earlier none of the chicks in this series showed syndactylysm and complete or even partial suppression of toes though selective shortening of the toes in cases of extreme micromelia has been reported by Landauer (1954).

The bending of femur, so commonly obtained with thallium treatment (Karnofsky *et, al*, 1950) is relatively rare in eserine experiments.

The frequency of occurrence of ectopia viscerae is approximately the same in control and experimental chicks and hence of no consequence in the present discussion.

The works of the various previous authors and the present one taken together indicate that the teratogenic agents contribute to the formation of not an unsimilar syndrome of abnormalities. In other words whatever may be the agents, the parts mainly affected are the skeleton of the hind limbs and the bones of the face. These agents, therefore, may be interfering with metabolic processes involved in the formation of the cartilaginious skeleton. That means, they partially obstruct one or more of the chemical chains, which progressing together, ultimately produce the normal skeletal framework.

Summary

1. The injection of eserine sulphate as a teratogenic agent into the air space of the egg has been deemed to have little interference with the nutrition of the embryo and was therefore tried in these experiments.

2. The data of experimentally produced abnormalities have been detailed and tabulated for comparison with the spontaneously occuring ones.

3. The close association between the facial and the limb abnormalities has been interpreted as metabolic relatedness of the two parts, atleast during the critical period.

4. The abnormalities and their probable origin in a defective laying down of the actively growing cartilaginous primordia have been discussed.

5. Since a very wide variety of agents produce a closely similar syndrome, it has been postulated that the drugs act by specific interference in any of the various metabolic chains.

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THE EXCRETORY SPHERIOLES IN THE DIGESTIVE GLAND OF *PILA VIRENS*

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IN what are usually described as excretory cells in the digestive gland of many gastropods there are present very conspicuous greenish brown bodies. These have been described by some authors as excretory spherioles. Morton (1952) Macmunn (1900) regarded these spherioles to contain chlorophyllous pigments derived from food substances and extracted from blood. But we have no information regarding their chemical composition or precise role.

The present account deals with the chemical composition of these bodies in *Pila virens*. I have examined also the excretory spherioles in other genera of molluscs like *Turritella and Paludomus*.

The shape of the spherioles varies to some extent in the different species and also with the age of the animal. They are periodically ejected from the cells and pass out to the exterior through the intestine and rectum. When the living stomach together with the attached digestive gland is opened and examined in normal saline solution under the binocular microscope, the spherioles can be seen coming out of the opening of the digestive gland. In a previous study (1954) it was observed that in specimens of *Pila virens* fed on iron saccharate iron is absorbed by the cells of the digestive gland and in prolonged feeding on this substance concentric layers of absorbed iron could be detected round the excretory spherioles by the ' prussian blue ' test. This suggests that the spherioles have an excretory role and help in the elimination of the absorbed iron.

Method of extraction of the spherioles

The separation of these bodies from the cells in the digestive gland presents some difficulties on account of their intracellular location. Crushing the digestive gland in a mortar and subsequently homogenising it in a tissue disintegrator was found to be the best method for extracting the excretory spherioles from the tissue. The homogenate containing the solid spherioles was taken in a test tabe and mixed with water, and after repeat-

ed decantation and centrifugation the supernatant fluid was completely drained off and the excretory spherioles dried and stored in a clean specimen tube. By this method all the excretory spherioles of the digestive gland could be extracted.

Chemical methods

The excretory spherioles were analysed chemically after ashing for iron, calcium, magnesium and uric acid. For the determination of iron the usual volumetric method of titration with potassium permanganate was adopted. Calcium was estimated after precipitation as oxalate and estimating the oxalate with permanganate titration. For the estimation of magnesium the eluent was precipitated after elimination of calcium as phosphate and the phosphate estimated colorimetrically. Uric acid was estimated according to the method of Benedict and Franke as described by Hawk *et al.* (1954). For each experiment, the excretory bodies from five animals were taken, as the quantity present in a single animal is too low for satisfactory chemical analysis. The excretory bodies in normally active animals as well as aestivating ones were studied.

Results

Table I shows the quantitative distribution of the excretory spherioles in active and aestivating animals. During aestivation there is a slight decrease in the total amount of the excretory spherioles which may not be very significant. The slight decrease may be due to a slight dissolution of these bodies in the lactic acid which is produced in considerable quantities in the tissues during aestivation. Further as the ultimate source of some of the components of the animal is the food of the animal which is lacking during aestivation, the slight decrease during aestivation can be understood.

Table II shows that during aestivation, the calcium as well magnesium content of the excretory bodies is lower while the relative iron-content is higher. But there is no change in the absolute value of iron-content. As the total weight of the bodies gets reduced during aestivation the percentage of iron appears higher.

The most important constituent of the excretory bodies is however, uric acid. Evidently complex urates make up a good portion of these excretory bodies. During active life 28% of the total weight of the

bodies is formed by uric acid, and during aestivation it is 25%. The reduction is evidently related to the reduction in the total quantity of these bodies during aestivation which has been explained before. Further my studies of aestivation (1956) show there is anaerobic glycolysis involving production of lactic acid in the tissues.

The excretory bodies may to a slight extent dissolve in the lactic acid. However, lactic acid in the excretory bodies could be detected only quantitatively, as the amount present is very low. During active life only a negligible quantity is present, but during aestivation there is a slight increase.

Conclusions

The occurrence of uric acid in the renal chambers of *Pila globosa* has been described by Saxena (1955) who however, did not estimate it in the digestive gland. My studies (1955) have shown that the nitrogen catabolism of *Pila virens* can alternate from ureotelism to uricotelism and *vice versa*, according to the demands of the environment. The present account shows that an appreciable amount of uric acid is deposited in the ' excretory spherioles ' which are eliminated to the exterior at intervals through the hind gut. The name ' excretory spherioles ' used by some authors finds a chemical justification in the present investigation.

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TABLE I

Quantitative distribution of excretory spherioles in the digestive gland

(Average of 3 sets of experiments—for each experiment the excretory spherioles from 5 animals were taken)

State of the animal	Average % of the excretory spherioles to the net weight of the digestive gland.
Active life	4.1 (4.0—4.2)
Aestivation (6 months)	3.7 (3.65—3.75)

TABLE II

Quantitative analysis of the metals of the excretory spherioles during active life and aestivation

(Average of 5 experiments—for each experiment 5 animals were taken)

Metal	Average percentage to the weight of the excretory spherioles	
	Active animal	6 months aestivation
Iron	4.2 (4.0—4.5)	5.3 (5.1—5.6)
Calcium	8.9 (8.6—9.1)	8.1 (8.0—8.2)
Magnesium	1.3 (1.1—1.4)	(1.0—1.1)

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URINARY EXCRETION OF NICOTINIC ACID IN TOXAEMIA OF PREGNANCY.*

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TOXAEMIA of pregnancy, forms a major problem to Obstetricians, all over the world. Maternal as well as foetal mortality, resulting from it, is a very common phenomenon. As a cause of maternal and foetal mortality, it ranks only next to anaemia, in this country. It is recognised that nutrition plays an important part in the causation of this condition. One must be concerned not only with the likes and dislikes of the sick person in regard to nutrition but also with the requirements peculiar to his condition. Facts revealing close interrelationships among nutrients, internal secretions and metabolism emphasize that nutritional diseases may be chronic or acute and may develop suddenly or insidiously. It is of greatest importante in this connection to know whether the dietary habits of the generality of the people are adequate for their nutritional needs. This is particularly important during pregnancy, when the growing foetus makes extra demands upon the woman. A great responsibility lies on the individuals who give preconceptional and prenatal medical care, not only to protect the immediate and future health of the woman, but also to protect the child to be born, from any metabolic imbalances and deficiencies, the residual effects of which might constitute a danger to the future health of that individual.

When mineral and vitamin intakes are low and mineral metabolism is defective during pregnancy, not only are the results a predisposition to bone and tooth defects in the foetus but such associated nutritional deficiencies in the mother may also be one of the etiological factors in vomitting, eclampsia etc. (Macy and Mack, 1954). King and Ride (1945) made a study of 370 cases of beri-beri complicating pregnancy, in which they considered that the primary factor responsible for the heavy increase in pregnancy toxaemia was a deficiency of vitamin B_1. They almost proved it by prophylactic and curative treatment by means of a diet rich in

* This paper forms a preliminary report of the work already completed before the I.C.M R. inquiry on Toxaemia of Pregnancy at the Medical College, Baroda, started. The work with regard to the other components of the Vitamin B Complex also has now been taken up.

this vitamin or by thiamine medication in dealing with these cases. Hobson (1948) in a dietary and clinical survey of pregnant woman with particular reference to toxaemia of pregnancy, found that the only deficiency in the diet common to all cases of toxaemia was in nicotinic acid. The mean intake of nicotinic acid was 2.0 mg. lower in the toxaemia group than that in the normal subjects. It therefore occurred to the author that deficiency of nicotinic acid or vitamin B_6 may be a predisposing factor in the production of toxemia of pregnancy. A study was therefore undertaken to find out the urinary excretory levels of nicotinic acid in patients with toxaemia of pregnancy, with a view to see if these findings can throw any light on the causative relationship between the deficiency of nicotinic acid and toxaemia of pregnancy. The results of this study are set forth here in this article.

Material and method

Fifteen cases of toxaemia of pregnancy admitted in the Maternity wards of S. S. G. Hospital, Baroda, have been studied. They ranged in their age from 18 to 39 years. In these cases, the clinical diagnosis of toxaemia of pregnancy was made if the blood pressure exceeded 140/90, where it had previously been normal or if albuminuria occurred. This appears to be the usual criterion adopted by many workers (Ebbs *et al* 1941; King and Ride, 1945). The samples of urine of these cases were obtained for analysis on their first admission in the Hospital wards.

Various methods have been suggested for the quantitative determination of nicotinic acid. These methods are based on the reaction in which the pyridine nucleus of the nicotinic acid is broken down by cyanogen-bromide and an aromatic amine to give a compound which is coloured yellow. The intensity of the colour which is a measure of the nicotinic acid content is determined colorimetrically.

In the present study, for the estimation of nicotinic acid in urine, aniline—cyanogen-bromide method was adopted (Swaminathan 1939; Banergee *et al* 1948). 24 hour's urine collection was made in bottles containing 20 c.c. of 50% sulphuric acid (Pai, 1955). An accurate and complete estimation of nicotinic acid in urine involves the conversion of its derivatives into the free acid before any colorimetric test can be applied. These can be readily hydrolysed to nicotinic acid by boiling with mineral acids or alkalis (Swaminathan, 1938). This method has slight advantages in that it removes the ammonia formed during hydrolysis.

The underlyiug principle of the method used in this investigation involves the steps which included extraction of the sample and decolorization under conditions which produce a minimum amount of interferring material and colour in the reaction of niacin, cyanogen-bromide and the aromatic amine (Vitamin assay 1951).

After the urine, taken in 25 cc. quantity, was hydrolysed with acid, it was treated with basic lead acetate and was adjusted to pH 10. After centrifugalization, the filtrate was treated with sulphuric acid in order to remove excess of lead acetate by precipitating as lead sulphate. Zinc sulphate was added to the filtrate and its pH was adjusted to 10. The filtrate obtained after centrifugalization again, was adjusted to pH 6.8 and an adequate quantity from it was used after adding phosphate buffer, for color development with cyanogen-bromide aniline reagent. The yellow colour obtained was matched in the photoelectric colorimeter with suitable reagent blank and the standard solution treated in the same way. This method was followed after obtaining the satisfactory results with the experiment of recovery % age of the vitamin.

Results

Below the results of the urinary excretion of nicotinic acid in 24-hours' sample have been tabulated in the tables and are represented in the graph.

TABLE 1

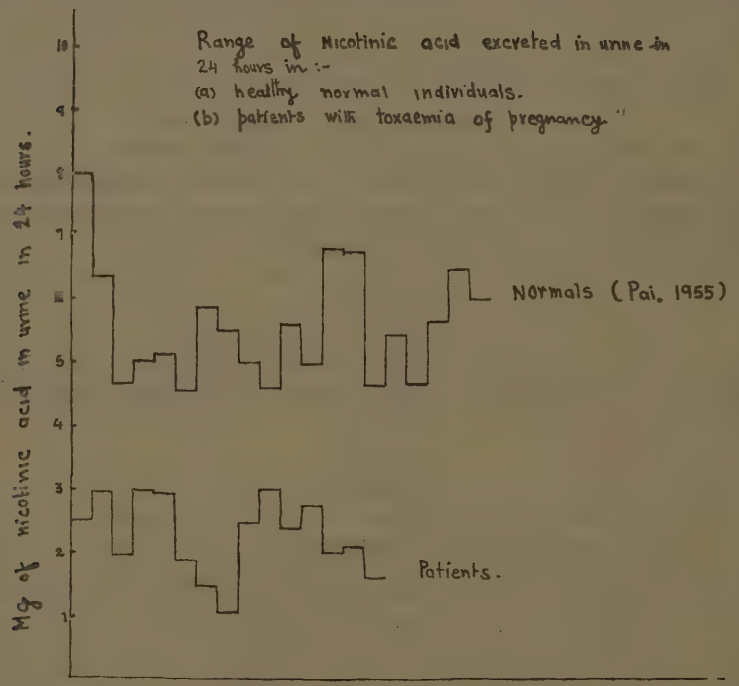
Urinary nicotinic acid excretion in mg. in 24 hours

Sr. No.	Patient	Nicotinic acid	Sr. No.	Patient	Nicotinic acid
1	N. R.	2.5	9	S. K.	2.48
2	K. S.	2.9	10	K. N.	3.0
3	N. Ram.	1.9	11	Sh. M.	2.4
4	P. S.	2.9	12	B. B.	2.46
5	V. B.	2.8	13	I. K.	2.0
6	L. S.	1.8	14	G. R.	2.2
7	S. M.	1.44	15	P. A.	1.65
8	S. P.	1.01			

TABLE 2

Average excretion of nicotinic acid in urine in 24 hours

	Nicotinic acid in mg.
Maximum	3.0
Minimum	1.01
Mean	2.24
Standard error	±0.17



Graph showing the urinary excretion of nicotinic acid in 24 hours' sample

The blood pressure of these patients ranged between 140/100 and 180/120 and albuminuria was found to be present in about 70% of the total number of cases. The nicotinic acid values ranged between 1.01 to 3.0 mg. per day, with the average 2.24 ± 0.17 mg. per day. These values have been compared with those for normal individuals previously reported (vide graph).

Discussion

There are a number of facts relating toxaemia of pregnancy to a deficiency of vitamin B_2 complex. This malady occurs commonly in association with pellagra and vitamin B deficiency. Thus Ross *et al* (1938) and Siddall (1940) had shown that there was a striking relationship of the distribution of toxaemia in the Southern States of U.S.A. with the distribution of Vitamin B deficiency and pellagra. According to the findings of King *et al* (1945), a high incidence of toxaemia and eclampsia was associated with a deficiency of the B group of vitamins. It was believed by Dieckmann (1938) that toxaemia was rare in Native African and other races except when they adopted the white man's diet; it was common amongst negroes in the U.S.A. In England, the Peoples League of Health (1942) showed a significant lowering in the incidence of toxaemia in a group of women kept on supplemented diets containing vitamin B_2 complex in addition to other vitamins.

A high incidence of toxaemia on poor diets, but none on good diets was found by Burke *et al* (1943). No significant difference was observed in the incidence of toxaemia in groups whose diets were supplemented with calcium, phosphorus, iron, vitamins A & D (Ross 1947; Ebbs *et al*, 1941 and Dieckmann *et al*, 1944). Many of the experiments which showed no significant change in the incidence of toxaemia in groups with supplemented diets made no allowance for the provision of the vitamin B_2 complex in the supplemented diets. The following remarks made by Ross (1947) in his presidential address to the South Atlantic Association of Obstetricians and gynaecologists may be quoted here in this connection, "We have rarely found toxaemia in the intelligent and adequately nourished group but it is the prime factor in the improperly nourished. In our areas such a patient would develop pellagra if exposed to the sun, and we feel, may develop symptoms of pregnancy toxaemia if she becomes pregnant. The patient that we see in eclamptic convulsions has come from the same group who subsisted on a diet similar to pellagrins". Duncan (1947) reported, after his experiences on the Yukon, that toxaemia was almost uncommon, the plausible cause of which he attributed to the diet consisting of larger quantities of meat and eggs.

Thus the literature quoted above reveals clearly the evidence of a very close relationship between a deficiency of vitamin B_2 complex and the occurrence of toxaemia of pregnancy. In the present series, the urin-

ary excretion of nicotinic acid in 24 hours' collection has been found to vary between 1.01 and 3.0 mg. with an average of 2.24 ± 0.17 mg/day. This range can be compared with that obtained in normal healthy individuals. In case of the normal healthy subjects the excretion of nicotinic acid in 24-hours' sample of urine was found to vary between 4.6 and 7.9 with an average of 5.56 ± 0.21 mg/day (vide chart). Thus the urinary excretion of this vitamin in the present cases of toxaemia of pregnancy is lower when compared with the normal urinary excretion and it is about slightly less than 50% of the latter. This lower urinary excretion of the vitamin in the cases of toxaemia of pregnancy also indicates that there possibly exists a close relationship between the deficiency of this vitamin, nicotinic acid and the occurrence of toxaemia of pregnancy. Of course, it must be pointed out here that the criterion taken in this study for judging the deficiency of the vitamin in the patients is its 24-hours' urinary excretion. Though this criterion will, to a large extent, be serving as an indication towards judging the deficiency of the vitamin concerned, still when supplemented by other tests like the saturation tests, the finding, will no doubt be giving added evidence in that direction. Further work on this line, not only with regard to nicotinic acid deficiency but also to deficiencies of other related vitamins of the B complex group, is in progress. And it is expected that more light will be thrown by this on this problem of toxaemia of pregnancy.

Summary

1. Fifteen cases clinically diagnosed as toxaemia of pregnancy have been studied for their 24 hours' urinary excretion of nicotinic acid.

2. The range of nicotinic acid excreted in 24 hours' collection of urine in the patients was found to be varying between 1.01 and 3.0 mg. with an average of 2.24 ± 0.17 mg. per day.

3. This mean value for the excretion of nicotinic acid in 24 hours' urine sample, when compared with that obtained in the normal healthy individuals, which had been previously reported from this laboratory, is found to be much lower than the latter.

4. The excretion of the vitamin in the patients comes to be about slightly less than 50% of that in the normal healthy individuals.

5. The results seem to indicate that there probably exists a close relationship between the deficiency of nicotinic acid and the occurrence of toxaemia of pregnancy.

6. These findings have been compared with those of other workers and the significance of the results has been discussed.

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THE ALKALINE PHOSPHATASE ACTIVITY IN THE GILLS OF FRESHWATER MUSSEL *POTAMIDA (PARREYSIA) CORRUGATA*

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IT has long been known that freshwater mussels carry their young in the gills, which function as brood pouches until the completion of the embryonic development and liberation of glochidia. The gills into which the eggs are received from the suprabranchial chamber are known as marsupia.

The morphological differences between the marsupial and nonmarsupial gills have been investigated by several authors, particularly Ortmann (1910), Churchill (1912). The marsupial gills of the Indian species have been described by Prashad (1918 a & b).

I have been studying the possible biochemical differences underlying the morphological differences of the male and the female gills. The present account deals with the alkaline phosphatase activity in the gills of *Potamida (Parreysia)*. In this form both the outer gill and the inner gill in the female function as marsupia in the breeding season.

Material and methods

The outer and inner gills in the male and in the non-gravid female were investigated. Only the potential marsupia and not the gravid marsupia were taken, as the presence of embryos in the latter would interfere with the estimation of the alkaline phosphatase of the gills.

The chemical method of estimation of alkaline phospatase was preferred to the histochemical method.

For the extraction of the enzyme the method described by Mary and Kind (1952) was adopted. The gills were removed from the live animal and placed immediately in a tube containing 10 c.c. of chloroform saturated water (previously weighed) and the weight of the tissue noted. The tissue was then homogenised and the homogeneate stored in a refrigerator for 48 hours. At the end of this period it was centrifuged and the clear supernatant fluid used as the enzyme preparation. For determining the activity of the enzyme the procedure described by Hawk *et al.* (1954) was adopted, using sodium glycerophosphate as substrate. 9 c.c. of the substrate solu-

tion was taken in a glass stoppered bottle and placed in an incubator at 37°C and given sufficient time to warm upto that temperature and remain in equilibrium. 1 c.c. of the enzyme preparation was added, the time noted and incubation was allowed exactly for two hours. At the end of this period 2 ml of 30% trichloroacetic acid was added to stop the enzyme action and the incubated sample cooled in ice. It was then filtered through low ash filter paper. A control sample was prepared by following the same procedure but without allowing any time for incubation, the addition of 1 ml. of the enzyme sample being immediately followed by the addition of trichloroacetic acid and cooling in ice. A similar method was adopted for running a standard with the difference that 8 ml. of standard phosphate solution was employed. A fourth containing 8 ml. of 5% T.C.A. served as blank.

For colour development 8 c.c. from each tube was measured into tubes graduated at 10 ml. 1 ml. of molybdate II reagent and 0.4 ml. of 1-2-3-4-aminonaphthol sulphonic reagent added and the final volume made up to 10 ml. 5 minutes were allowed for colour development and the intensity of colour measured photometrically with the photoelectric colorimeter (Lumetron), using filter 660 μ. The difference in the reading of the incubated and that of the control samples represents the amount of inorganic phosphate released by the enzyme.

The results are shown in Table I. It will be seen from the table that the alkaline phosphatase activity in the female gills is consistantly higher than in the male gills. In both the male and the female the outer and inner gills show a close similarity of alkaline phosphatase activity. The female gills, although belonging to non-gravid specimens, were in different stages of growth. So they show a wider range of variation in the alkaline phosphatase activity.

It is well known that the phosphatases are vital not only for the function of bone, but also for the metabolism of carbohydrates, nucleotides and phospholipids, besides muscular contraction. They are commonly found in the cytoplasm of growing and secreting cells in which protein synthesis is going on. The consistent difference between the potential marsupial gills and the male gills in the alkaline phosphatase activity is significant. One of the differences between the marsupial gill and the respiratory gill is the increased thickness and folding of a glandular epithelium in the former, as Ortmann (1910) and Churchill (1912) have

xplained. This glandular epithelium may be related to the conglutina-
.on and protection of the embryos after they arrive in the water tubes of
he marsupium. The potentiality of the gills of the female to develop into
aarsupia is evidently related to the greater alkaline phosphatase activity
n them as compared with the respiratory gill of the male.

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TABLE I

Alkaline Phosphatase activity in mgms. of phosphorus per grm. of tissue released in 2 hours at 37°C.

Expt. No.	Male outer gill	Female outer gill	Male inner gill	Female inner gill
1	0.51	0.93	0.50	0.98
2	0.49	1.16	0.54	1.16
3	0.59	0.93	0.52	0.96
4	0.49	1.41	0.49	1.42
5	0.50	0.86	0.53	0.88
6	0.58	0.94	0.56	0.94
Mean	0.51 (± .00012)	1.038 (± .25)	0.52 (± .003)	1.073 (± 0.13)

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ON THE OCCURRENCE OF LIPASE IN THE SKELETAL MUSCLES OF VERTEBRATES AND ITS POSSIBLE SIGNIFICANCE IN SUSTAINED MUSCULAR ACTIVITY

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THAT fat and not glycogen forms the chief fuel in many flying animals in long and sustained flight involving intense muscular activity is aining increasing support. The high energy value and the property of eing easily stored in tissues are certain positive advantages of fat for eing used as fuel, particularly when a continuous flow of energy for a long ime is needed. Since 1920 considerable amount of work has accumulated emonstrating an appreciable reduction in the muscle fat during prolonged xercise and thereby denoting the possibility of the utilization of fat uring such muscular activity. The whole literature on this subject has een reviewed by Weis Fogh (1952) and George and Jyoti (1955$_a$ and '55$_b$) nd so it is not necessary to cite them again here. Weis Fough showed hat in the locust, *Schistocerca gregaria*, atleast two-thirds of the energy berated during flight was derived from fat. His estimations also showed hat per locust about 60 mg. of fat and 25 mg. of other materials chiefly lycogen were metabolized. George and Jyoti (1955$_b$) found that there is onsiderable reduction in the lipid content of the breast muscle of good iers during activity, when the animal is forced to fly for a long time or vhen the muscle of the pithed animal is continuously stimulated electrically, the extent of reduction varying according to the duration of the xercise. They (1955$_a$) further observed a reduction in the number of fat lobules in the fat loaded narrow fibres of the exercised *pectoralis major* uscle of the pigeon, using proper histological techniques. Recently eorge and Jyoti (1956-unpublished) have shown that when a pigeon is ubjected to forced flight till it is completely fatigued, about 78% of the nergy utilized is derived from fat while only about 22% from glycogen.

If therefore fat is used as fuel in muscular activity the splitting up f fat into fatty acids and glycerol should be the first step in the process. atty acid is then oxidized to carbon dioxide and water through -oxidation and the citric acid cycle. Evidence of such oxidation in ammalian liver cells with the mitochondria as the centres of such action

is available from the work of Kennedy and Lehninger (1948). The recent researches of David E. Green and his collaborators (Green-1953, Mii and Green-1954, Green-1955) have confirmed beyond doubt that the complex enzyme system in the mitochondria of the cells of the liver, heart and kidney can bring about such oxidation. In view of these findings it is reasonable to expect that the oxidation of fatty acids takes place in the mitochondria of muscle cells also. Partial oxidation of fatty acids in the liver leads to the production of ketone bodies which are completely oxidized to carbon dioxide and water in the muscles (Fruton and Simmonds-1953, Harrow-1950). It has also been shown that extrahepatic tissues can effect direct oxidation of long chain fatty acids to carbon dioxide and water through the citric acid cycle without giving rise to ketone bodies (Harrow-1950, Fruton and Simmonds-1953).

Having been thus convinced of the possibility of the oxidation of fatty acids in muscle cells in the process of fat utilization during prolonged and intense muscular activity, we considered it worthwhile looking for an enzyme which could hydrolyze fat into fatty acids and glycerol which is really the first step in the utilization of fat.

The presence of lipase in blood, liver and pancreas of vertebrates, in the intestinal tissue of many invertebrates, and, in seeds and moulds among plants is well known. But very little however is known regarding its presence in vertebrate tissues other than those mentioned above. The work of Schmidt Nielson and Stene (1939) who noted lipase in the cooked muscle of salmon is the only important work on this subject we are aware of.

In our present investigation we conducted experiments to see whether there is a true lipase in the *pectoralis major* muscle of the rock pigeon (*Columba livia*), and extended this study to the *pectoralis major* and *gastrocnemeus* muscles of, the domestic fowl (*Gallus domesticus*) and also in the latter muscle of the frog (*Rana tigrina*).

Detection of Lipase Activity

Materials

Preparation of the Enzyme Material

Portions of the *pectoralis major* muscle from pigeon which was bled to death by cutting off the head and the pectoral blood vessels, were cut out and immediately frozen in an ice-acetone bath. The extraction

medium used was 70% glycerine which was cooled to 0°C. The muscle was then quickly cut into small pieces, crushed and ground to a fine pulp in a mortar which was also previously cooled to 0°C and kept in an ice bath. Glycerine was slowly added to the pulp, well slaked and allowed to stand for a while. The glycerine soluble fraction was then separated out by squeezing through a cheese cloth. A few drops of tuloune were added to the clear filtrate to prevent putrifaction and kept at 0°C in a refrigerator. By this method even though all the enzyme in the muscle might not be extracted, a considerable part of it atleast should get dissolved, since glycerine is a good solvent for lipases. This crude glycerine extract was used as the enzyme material in all the experiments.

Substrates

For determining the activity of the extract four substrates—two triglycerides *viz.* tributyrin and triacetin and two vegetable oils castor oil and olive oil—were used.

Methods

The following methods were employed.

(1) The method of Cherry and Crandall (Cherry and Crandall-1932) for determining the lipase activity in blood serum using olive oil as substrate.

(2) Lipase determination with the aid of polyvinyl alcohol (Fiore and Nord-1949) using all the substrates mentioned above.

(3) Method for lipase (Tributyrinase) determination (Goldstein, Epstein, and Roe-1948) using tributyrin as substrate.

(4) Spectrophotometric method of lipase activity determination (Herr Jr. and Sumner-1955) using castor oil as substrate.

In all these assays a boiled extract was used as the control with all the other conditions remaining the same. The reaction mixtures were incubated at 40°C and pH maintained at 7 in all the experiments.

Results

In the titrimetric methods 1, 2 and 3 an increase in the acidity and in method 4, a decrease in the optical density of the reaction mixture, indicated the presence of lipolytic activity.

Comments

In the titrimetric methods the determination of the end point was somewhat difficult owing to precipitation. In order to verify the accuracy

of the end point in titration the pH of the titrated material was later determined by electrometric methods. In the case of the polyvinyl alcohol method, the alternate procedure recommended by Fiore and Nord (1949) was found to be quite suitable in judging the end point.

Polyvinyl alcohol however was not found to be a very efficient emulsifying medium for certain of the substrates. Tributyrin and castor oil did not yield emulsions at all. But when one or two drops of Tween 80 were added, these substrates formed a fine emulsion in the Waring Blendor. It should be mentioned here that although Tweens themselves are considered to be substrates for lipase, there is no detectable activity at a low concentration such as two drops in 100 ml. of polyvinyl alcohol solution.

Quantitative Determination of Lipase Activity

Method and Materials

Of all the methods employed in the above experiments, the one using polyvinyl alcohol was found to be the most convenient. This method with certain modifications was therefore adopted for all the subsequent studies. Tributyrin was used as the substrate because this yielded the best results in the earlier experiments.

Preparation of the Polyvinyl Alcohol Solution

2.5 gm. of polyvinyl alcohol (Grade 71-30) was shaken in an Erlenmeyer flask with 250 ml. of distilled water. 1.25 ml. of 0.1N HCl was then added and the mixture heated at 75-85°C for a few minutes, till the solution became clear, when 25 ml. of distilled, water was added, and the whole heated for some more time. The solution was then cooled and brought to the desired pH by adding 0.1N NaOH. Table I shows the amount of alkali added for different pH.

Preparation of the Emulsion

To 100 ml. of the polyvinyl alcohol solution (PVA) were added 2.8 ml. of tributyrin and a single drop of Tween 80 and emulsified in a Waring Blendor for five minutes and was used after some time.

Buffer Solution

A McIlvaine buffer of the same pH as that of the polyvinyl alcohol soln. was used.

Preparation of the Enzyme Material

Glycerine extracts of different concentrations were prepared. In each case 100 ml. of 70% glycerine was used for extraction and the quantity of muscle in gm. taken were in multiples of ten. Thus four samples with 10, 20, 30 and 40 gm. of muscle were extracted with 100 ml. of glycerine in each case. In all the studies except that on the effect of the concentration of the enzyme, an extract with a concentration of 40 gm./100 ml. glycerine was used.

Procedure

Into a 250 ml. Erlenmeyer flask were measured 10 ml. of the emulsion, 5 ml. of the buffer and 5 ml. of the enzyme preparation. The mixture was shaken gently and incubated at 40°C for the desired length of time with constant shaking. For all our studies the incubation period was fixed at 4 hrs. except where it is stated otherwise.

At the end of the incubation period 30 ml. of a 1 : 1 alcohol-acetone mixture was added to stop further enzymic activity. After 10 minutes it was filtered using a fluted No. 1 Whatman filter paper and 25 ml. of the clear filtrate was titrated against 0.05N aqueous NaOH using a microburette. 0.3 ml. of a 1% soln. of alcoholic phenolphthalein was used as an indicator. The value thus obtained was doubled and recorded as such.

Control

Two controls were tried. In one the enzyme was boiled for about ten minutes and used after cooling. In the second, the fresh enzyme preparation was used after the addition of the alcohol-acetone mixture to the substrate, and thereby breaking up the emulsion and rendering the enzyme completely inactive. The readings obtained for these two types of control experiments with the heat inactivated as well as the alcohol-acetone inactivated enzyme preparations were found to be identical. The second method of inactivation was employed in our study because it was found to be more convenient.

Results

The results obtained were as follows :—

pH Optimum

Experiments were carried out using reaction mixtures of various pH. McIlvaine buffer of a pH range of 2 to 8 was used. For pH 9, a 0.2M solution of disodium hydrogen phosphate was employed. Lipolytic activity was found to be the highest at pH 8 as can be seen from Table I and also from the graph (Fig. 1).

TABLE I

Showing the lipolytic activity of the muscle extract at various pH

pH	Amount of 0.1N HCl added to PVA solution (in ml.)	Amount of 0.1N NaOH added to PVA solution (in ml.)	Amount of 0.05N NaOH titrated (in ml.)		Difference
			Control	Sample	
4	1.30	—	25.2	29.0	3.8
5	1.25	—	21.2	25.6	4.4
6	,,	1.375	17.9	25.3	7.4
7	,,	1.5	12.6	24.4	11.8
7.6	,,	1.6	8.9	24.0	15.1
8	,,	1.7	7.4	23.6	16.2
9	,,	1 9	7.2	22.3	15.1

Effect of Temperature

The enzymic activity of the extract was studied at different temperatures ranging from 0°C to 60°C, keeping the pH constant at 7.6, which is nearest to the pH of the avian blood. Since time and temperature are interdependent variables it was decided to conduct three sets of experiments with different periods of incubation. It was found that for shorter periods of incubation, the temperature optimum was 50°C, whereas the activity is reduced considerably at this temperature when incubated for longer periods, for which the optimum was at 40°C. The results obtained in this study are given in Table II and illustrated in fig. 2. It is clearly seen that the enzyme is quite stable at higher temperatures and is less active at lower temperatures.

TABLE II

Showing the effect of temperature on the enzymic activity at different periods of incubation

Incubation period in hours	Amt. of 0.05N NaOH in ml. equivalent to acid liberated at different temperatures						
	0°C	15°C	30°C	35°C	40°C	50°C	60°C
1	0.4	0.4	1.2	2.9	4.2	5.0	3.8
6	0.6	2.2	10.1	12.4	13.4	15.4	12.5
12	1.7	6.6	14.4	16.4	18.3	15.5	14.4

Time Factor in the Enzymic Activity

Several samples were run for varying lengths of time, all other conditions such as the pH, temperature and the concentration of the substrate

and the enzyme material remaining the same. The pH was adjusted at 7.6. It can be seen from Table III and fig. 3 that the initial velocity of the reaction decreases with time.

TABLE III

Showing the activity rate at different periods of incubation

Sample	Time in hours	ml. 0.05N NaOH equivalent to acid liberated
I	1	4.20
II	2	6.40
III	$3\frac{1}{2}$	13.10
IV	7	17.30
V	$10\frac{1}{2}$	18.60
VI	14	23.10
VII	$17\frac{1}{2}$	23.34

Effect of Concentration of the Enzyme

As mentioned above four different concentrations of the enzyme were prepared for this study. The activity is found to vary accordingly (Table IV and Fig. 4). The samples were incubated for a period of 14 hrs. and at pH 7.6.

TABLE IV

Showing the effect of concentration of the enzyme

Sample	Concentration of the extract gm. muscle/100 ml. glycerine	ml. 0.05N NaOH equivalent to the butyric acid liberated
I	10	8.05
II	20	10.80
III	30	14.15
IV	40	21.15

Action on Different Substrates

Experiments using different substrates were carried out at pH 8 which is the optimum for this enzyme at 40°C. Four substrates *viz.* tributyrin, triacetin, olive oil and castor oil were tested. The rate of hydrolysis of these substrates come in the following order tributyrin > triacetin > castor oil > olive oil (Table V).

TABLE V

Showing the enzymic action on different substrates

Substrate	Substrate conc. ml./100 ml. PVA	ml. 0.05N NaOH titrated		
		Control	Sample	Difference
Tributyrin	2.8	7.4	25.2	17.8
Triacetin	1.9	8.1	21.2	13.1
Castor oil	3.0	7.5	10.0	2.5
Olive oil	3.0	7.7	8.8	1.1

Definitions

For the measurement of lipase activity and for the purpose of comparison of the activity in different muscles it was considered necessary to adopt a certain standard. The standard we have adopted for this study is expressed as lipase units and can be defined as follows :

Lipase Unit

The amount of lipase required to liberate that quantity of butyric acid equivalent to 1 ml. of 0.05N NaOH, when 10 ml. of the substrate, of the concentration, 2.8 ml. tributyrin in 100 ml. polyvinyl alcohol solution and emulsified with one drop of Tween 80, is allowed to react with 5 ml. of a McIlvaine buffer of pH 8 at 40°C for 4 hrs. the total volume of the reaction mixture being 20 ml.

Lipase Value

The number of lipase units present in one gm. of wet muscle.

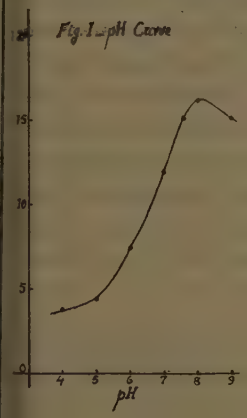
Calculation

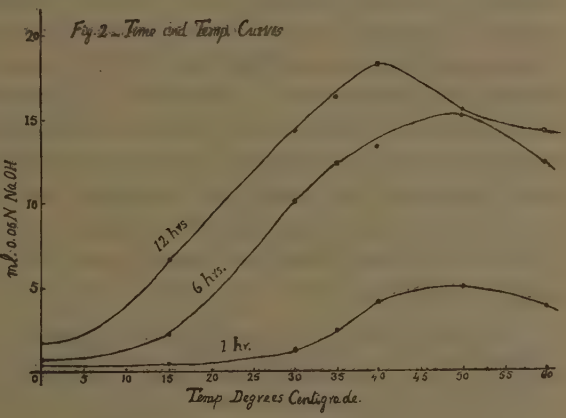
5 ml. of an extract of 40 gm. of the *pectoralis major* of pigeon in 100 ml. glycerine liberates butyric acid equivalent to 17 ml. of 0.05N NaOH on the average under the above conditions. The number of lipase units in 5 ml. of the extract is therefore 17. Five ml. of the extract contains 2 gm. of muscle with 17 units of lipase in it. So, the lipase value of the *pectoralis major* muscle of pigeon is 8.5. Table VI shows the lipase value of the different muscles of the three animals examined.

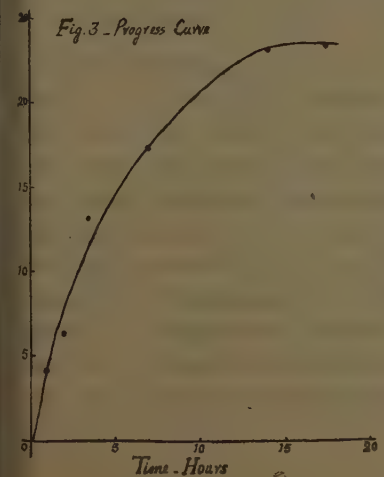
TABLE VI

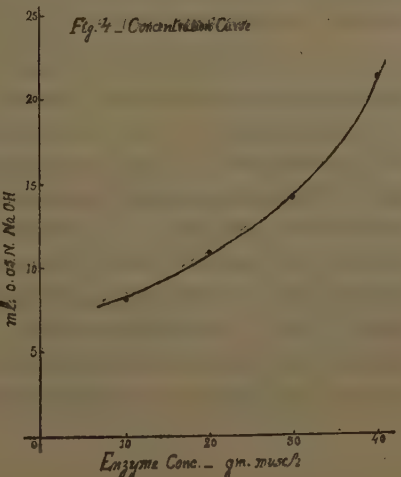
Showing the lipase value of different muscles

Name of the animal	Name of the muscle	Conc. of the enzyme gm. muscle/100 ml. glycerine	Lipase units in 5 ml. of the enzyme material	Lipase value
Pigeon	*Pectoralis major*	40	17.0	8.5
Fowl	,,	,,	2.0	1.0
,,	*Gastrocnemeus*	,,	2.2	1.1
Frog	,,	,,	1.6	0.8









graphs illustrating the activity of the lipase of the *Pectoralis major* muscle of the pigeon.

Discussion

That the active substance present in the muscle extract is an enzyme is evinced by the fact that it satisfies the requisite conditions for being considered an enzyme in that it shows a progressive activity at increased periods of incubation (Table III and Fig. 3), a marked difference in the activity as the concentration is altered (Table IV and Fig. 4) an optimum pH (Table I and Fig. 1) and temperature (Table II and Fig 2). It is well known that muscle contains several oxidizing and hydrolysing enzymes. The hydrolysing enzymes so far known in muscle are of the nature of esterases. Lipase is essentially an esterase, but is distinguished from other esterases by the fact that it readily hydrolyses lipids, which are esters of organic acids with alcohol, especially triglycerides in preference to any other substrate (Baldwin, 1953 ; Fruton and Simmonds, 1953 ; Sumner and Somers '53). Tributyrin and triacetin are triglycerides of butyric and acetic acids respectively, and are usually used as substrates in the study of the known lipases, such as of the liver, pancreas, castor bean and fusarium. The hydrolytic activity of the muscle extract on these substrates is considerable and other substrates like olive oil and castor oil are also acted upon though to a lesser extent. The enzyme in the muscle extract therefore is to be regarded as a true lipase.

The possible physiological role of such an enzyme in hydrolysing fat into fatty acids and glycerol during sustained muscular activity has already been pointed out. The amount of lipase present in a muscle should therefore be an index of the extent of possible fat utilization. The utilization of fat in the muscle could thus be directly correlated with its activity. George and Jyoti (1955_a, $'55_b$) have shown that there is a greater fat store and also a greater measure of reduction of fat during activity in the breast muscles of those birds where the narrow fat loaded fibres predominate over the broad glycogen loaded ones. It is therefore natural to expect a greater lipase activity in those muscles where the narrow fibres predominate and our observations also support this view. Thus the *pectoralis major* muscle of the pigeon which consists predominantly of narrow fibres has a high lipase value of 8.5, while that of the fowl consisting of only the large variety is only 1. In contrast to the pigeon breast muscle, the breast muscle of the fowl consisting of the broad glycogen loaded variety of fibres contains very little fat and when exercised electrically, the reduction of fat is extremely small (George and

Jyoti-1955$_b$). In the fowl again, the fat content and its reduction during exercise in the more active leg muscle is greater than that of the breast muscle (George and Jyoti-1955$_b$). The lipase value of 1.1 for the fowl leg muscle obtained in our experiments is slightly higher than that for the fowl *pectoralis major*. These observations regarding the fat content and its reduction during activity in the above muscles and their corresponding lipase values tend to show a relationship between the lipase content and fat utilization during sustained muscular activity.

Niemierko in 1929 and Buchwald and Cori in 1931 (as cited by Heilbrunn, 1952) noted a reduction in the fat content in the frog's leg muscle as well after prolonged stimulation. Our findings regarding the occurrence of lipase (lipase value 0.8) in the frog leg muscle also, not only provides an explanation for the hydrolysis of fat in the process of fat utilization in this muscle, but they also raise an important question as to whether lipase is present in all vertebrate muscles and perhaps in invertebrate muscles too. Such a possibility has been suggested by Baldwin (1953) when he wrote, "It has usually been assumed, though never proved, that fats are hydrolytically split into glycerol and free fatty acids before any oxidation takes place. This is not entirely an unreasonable supposition, for cells of most kinds seem to be furnished with lipolytic enzymes, the action of which is freely reversible." The results obtained in the present study support this suggestion atleast as far as the muscle tissue is concerned and the general conclusion that could be drawn is that the lipase concentration in the muscle would depend upon the extent of fat utilization in the muscle during activity.

Summary

(1) The *pectoralis major* muscle of the pigeon contains a true lipase, the pH optimum of which is 8.

(2) The enzyme shows only slight activity at temperatures below 15°C. The optimum temperature for long period of incubation is 40°C. It is found to be more stable than any other known lipase between temperatures 40° C and 60° C.

(3) The activity of the enzyme at different periods of incubation and at various concentrations is presented.

(4) Its activity on the different substrates studied has the order tributyrin > triacetin > castor oil > olive oil.

(5) The lipase value of the *pectoralis major* muscle of the pigeon is estimated to be 8.5.

(6) The occurrence of lipase is also noted in the breast and leg muscles of the fowl and in the leg muscle of the frog. The lipase values obtained for these muscles are 1.0,1.1, and 0.8 respectively.

(7) The significance of the occurrence of lipase in muscles is discussed.

Acknowledgement

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SOME OBSERVATIONS ON THE RANGE OF FLIGHT OF THE COMMON INDIAN HOUSEFLY, *MUSCA DOMESTICA NEBULO*, FABR.

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ARNOLD (1906, recorded by Niven, 1906) seems to have made the first experimental attempt to investigate the range of flight of the housefly, *Musca domestica*, L. He released 300 flies, each marked by an enamel spot on the thorax and recovered 5 of them at distances varying from 30 to 190 yards. Copeman *et al* (1911) made a number of experiments at Postwick. Several thousand flies sprinkled with chalk powder were liberated, and a few were recovered at distances varying from 300 to 1700 yards. Hine (1910, cited by Howard, 1911) released about 350 flies, marked with gold enamel paint and recovered some of them at a distance of 240 yards. Hewitt (1912) marked the flies by spraying them with a solution of rosolic acid in 10% alcohol. He released such marked flies and recovered some of them in three lots at distances of 520, 600 and 700 yards respectively. Nuttall *et al* (1913) found that the maximum range of flight in congested localities was about a quarter of a mile. They also observed that the height from which the flies are liberated, the atmospheric conditions (such as wind, temperature and weather) and the time of the day at which they are released are some of the factors which influence the dispersal of flies. Parker (1915, 1916) performed the same type of experiments and he could recover marked flies at distances varying from 50 to 3500 yards. Derbeneva Ukhova (1942) however, found the range of flight of *Musca domestica*, L. in the province of Archangel to be usually more than 350 yards.

No noteworthy attempts have been made in India, to find out the range of flight of the common Indian housefly, *Musca domestica nebulo*, Fabr. It was therefore considered desirable to obtain information on this point with reference to local conditions and a few experiments were carried out in Poona.

Material and Methods

As mentioned above houseflies used for the experiments were *Musca*

domestica nebulo, Fabr. from a homogeneous pure culture bred by the author in Poona. They were counted as they entered the wire balloon traps from the breeding cages and were marked by dusting thoroughly with red chalk powder. The process of dusting was repeated two or three times so that sufficient chalk was deposited on the flies. Only conspicuously marked flies were used for experiments.

1000 flies thus coloured were taken to the site selected for their liberation and they were allowed to escape from the trap. They were recovered later, on three successive days by netting from round about the localities. The distance in straight line between the point of release and the point of collection was measured, to ascertain the approximate distance travelled by them. In all two centres were selected for these experiments; one in a thickly populated city area and the other in Shivaji Nagar which is thinly populated and has plenty of open spaces. The experiments of releasing marked flies were repeated alternately at each centre. Temperature and relative humidity were recorded at the time of releasing the flies. Below are given the details of the attempts made.

Expt. No. 1; (18th-21st June, 1955); and Expt. No. 3; (2nd-5th July, 1955).

1000 marked flies each time were liberated from the roof of a house, near a market in the city area, at 10 A.M. on 18th June, 1955 and on 2nd July, 1955. The weather was warm and a light wind was blowing. The flies were collected on three successive days in the surrounding localities all over. They were recovered at distances ranging from 150 to 350 yards.

Expt. No. 2; (24th-27th June, 1955) and Expt. No. 4; (9th-12th July, 1955).

1000 marked flies each time were liberated from an open ground in the Municipal Colony, Shivaji Nagar, at 10 A.M. on 24th June, 1955, and on 9th July, 1955. The weather was warm and the wind was blowing heavily. They were collected on three successive days in the surrounding localities, and were recovered at distances ranging from 400 to 600 yards.

Results

The table given below summarises the results of the two sets of experiments.

TABLE I

Table giving results of experiments on the range of flight of *Musca domestica nebulo* Fabr. performed at Poona

	Dates of the Experiment	Weather conditions	Time of Liberation Temp. and Rel. Humidity	Colour used	No. of flies released	Date of recovery	No. of flies recovered at the maximum distance	Approximate distance in yards in straight line between point of release and point of recovery
1	18th June, 1955 (released from a residential building in the city)	Warm and Bright	10 A.M. 86°F. 66% R.H.	Red chalk powder	1,000	19th June '55	14	200 Yards
						20th June '55	12	300 ,,
						21st June '55	6	350 ,,
2	24th June, 1955 (released from an open plot at Shivaji Nagar)	Warm, bright and windy	10 A.M. 85°F. 64% R.H.	Red chalk powder	1,000	25th June '55	14	400 Yards
						26th June '55	8	550 ,,
						27th June '55	2	400 ,,
3	2nd July, 1955 (released from a residential building in the city)	Warm and bright	10 A.M. 85°F. 65% R.H.	Red chalk powder	1,000	3rd July '55	16	150 Yards
						4th July '55	9	300 ,,
						5th July '5	2	250 ,,
4	9th July, 1955 (released from an open plot at Shivaji Nagar)	Warm, bright and windy	10 A.M. 81°F. 70% R.H.	Red chalk powder	1,000	10th July '55	12	450 Yards
						11th July '55	6	500 ,,
						12th July '55	1	600 ,,

Discussion

The range of flight found in experiments 1 and 3 is between 150 to 350 yards, whereas in the other two experiments it is seen to be more, *i.e.* between 400 to 600 yards. The probable reason for this variation appears to be the difference between the ecological conditions prevailing in the two centres of release.

Experiments 1 and 3 were performed in a residential building in the city. The locality is thickly populated with buildings close to each other. It is a congested area with a market which is about 300 yards away from the building. Naturally the flies were attracted by odours from food-stuffs in the market and were thus collected there.

Experiments 2 and 4 were performed on an open plot in the Municipal Colony, Shivaji Nagar. This locality is comparatively cleaner and the residential buildings are fairly far away from each other. It being an open area, the wind blows rather heavily. The flies are thus subjected to currents of air. The flies were released from the open plot where no food material was present and naturally they were attracted by the odours from the sources of food, from the buildings round about, some of which were tea-shops. Thus, some of the flies were recovered near the tea-shops on the road, which is approximately 600 yards away from the open plot, from where they were released. Other flies were collected from the houses round about the open plot. The minimum distance travelled by flies in this area seems to be 400 yards.

It is presumed that released flies gather in the nearest situation where food material is easily available and which is suitable for oviposition. Once they find such a place they do not leave it. They do not seem to have any other incentive in their parambulation and the range of flight is limited to the discovery of such areas.

Summary

1. In order to assess the range of flight of houseflies, a thousand individuals were released at a time from two centres in Poona, one a thickly populated, congested locality and the other a thinly populated and cleaner one. In all four releases were made two from each of the centres.

2. The range of flight varied from 150 to 350 yards in the congested locality and from 400 to 600 yards in the thinly populated one.

3. The probable cause of the difference in the range of flight is discussed.

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THE AMINO ACIDS OF THE CRYSTALLINE STYLE OF *SANGUINOLARIA DIPHOS*

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SEVERAL authors as the appended list of references will show have studied the functions of the crystalline style of bivalves and gastropods, but relatively little work has been done on its chemical composition. Recently Hashimoto, Sato and Miyamoto (1954 & '55) have shown that the greater part of the style protein is a protein moiety of the nature of 'mucin' combined with uronic acid, gluco-samine, galactose, mannose, xylose and fucose and an unknown sugar. This finding, as the authors have pointed out, makes it difficult to accept Yonge's (1932) assumption that the style is a protein of the type of globulin.

In connection with a study of the physiology of *Sanguinolaria* I have investigated the composition of its crystalline style, and my findings confirm generally that the style is mucoid in nature with the component sugar glucuronic acid. The present account deals with the amino-acids of the protein moiety, the detailed account of the sugars being reserved for a future communication.

Methods

For the determination of the free and protein-bound amino-acids circular filter paper chromatographic technique as described by Giri *et al*, (1952a) with Whatman paper Nos. 1 & 20 was adopted. n-Butanolacetic acid-water mixture (4 : 1 : 5) was used as the solvent and 0.2% ninhydrin as colour developing reagent.

The amino-acids were identified by running mixed chromatograms as described by Giri and Rao (1952) and by spotting both known and unknown samples in the same chromatogram. Multiple development, as described by the same authors, was adopted to identify amino-acids which failed to separate as distinct bands.

For the separation of free amino-acids fresh styles were dissolved in distilled water and the protein precipitated by the addition of an excess quantity of absolute alcohol. Then it was centrifuged and the superna-

tant liquid treated with an equal volume of chloroform, centrifuged again and the upper alcoholic layer alone used for chromatographic analysis.

For the study of the protein bound amino-acids the styles were dried and hydrolysed with 6N hydrochloric acid. After further treatment the residue was dissolved in distilled water to which a few drops of 10% isopropanol were added.

Results

Free amino-acids :—Thirteen free amino-acids separating in thirteen bands are present in the crystalline style of *Sanguinolaria*. But Tryptophane appears in a faint band. The free amino-acids are listed in the table.

Protein bound amino acids :—In the hydrolysate of the whole style eighteen amino-acids are present separating in fifteen bands. Leucine and isoleucine, and valine and methionine appear as two combined single bands without separation. By multiple development technique as described by Giri *et al.* (1952) the components of these bands have been identified and are listed in the table.

In the style of *Mactra* investigated by Hashimato *et al.* (1954) only 10 amino-acids are present. Of the amino-acids present in *Sanguinolaria*, leucine, isoleucine, valine, proline, arginine, asparagine, histidine, ornithine and lysine are lacking in *Mactra*. But what is more interesting is the occurrence of a large number of free amino-acids in the crystalline style of *Sanguinolaria diphos*. Hashimoto *et al.* (1954) have not reported the occurrence of free amino-acids in the style of *Mactra* investigated by them. Some of the free amino acids occurring in the style have also been found to occur free in the blood, kidney and rectum of *Sanguinolaria*, (Venugopalan, 1956). This would suggest that the free amino-acids of the style are probably eliminated products of the cells of style sac *i. e.* what are left over after the synthetic processes involved in the secretion of the style. The free amino-acids should be regarded as stated by Fisher (1954) not merely as " a nutritive tender for ready acceptance by tissues, but as a metabolic currency. "

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TABLE

Amino-acids of the style

Amino-acids	Free amino-acids	Protein bound amino-acids
Leucine	+	+
Isoleucine	—	+
Phenylalanine	+	+
Valine	+	+
Methionine	—	+
Tryptophane	+	—
Tyrosine	+	+
Proline	+	+
Alanine	+	+
Threonine	—	—
Glutamic acid	+	+
Unidentified	+	—
Hydroxyproline	—	—
Glycine	+	+
Serine	—	+
Aspartic acid	—	+
Arginine	+	+
Asparagine	—	+
Histidine	+	+
Ornithine	—	+
Lysine	—	+
Cystine	+	+

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